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(54) Title: A METHOD FOR IDENTIFYING MICROORGANISMS, AND AIDS USEFUL THEREOF

(57) Abstract

Identification of a microorganism, in particular a bacterium, present in a sample. Nucleic acid (DNA or RNA) of the microorganism present in the sample is subjected to PCR or a different nucleic acid amplification method, utilizing one or more sets of universal primers based on a gene of the microorganism to be identified which comprises both conserved and variable regions, in particular the 16S rRNA gene, the primers being chosen in conserved areas which enclose a variable region. The product of the amplification is brought into single-stranded form, if necessary, and subjected to electrophoresis by which single-stranted nucleic acids of equal lengths can be separated from each other on the basis of differences in nucleotide sequence. The electrophoresed nucleic acid is detected and its position compared with those of a set of reference nucleic acids of known microorganisms. Set of suitable aids for practicing the method.

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Title: A method for identifying microorganisms, and aids useful therefor

FIELD OF THE INVENTION

The invention lies in the field of the detection and identification of microorganisms and relates to a method for demonstrating and identifying microorganisms in a sample, and to aids for use in such method.

PRIOR ART

The classic method for demonstrating and identifying microorganisms, in particular in clinical samples, such as urine, feces, sputum and blood, as well as in foods, takes place through culture in enrichment and selective media, followed by biochemical identification.

Sometimes the biochemical identification which follows the culturing steps is replaced by an immunological identification with the aid of antibodies, such as in an Enzyme Linked Immuno Sorbent Assay (ELISA) or an Enzyme Immuno Assay (EIA), or by a genetic identification with the aid of bacterial species-specific DNA probes in a hybridization analysis or bacterial species-specific primers in a polymerase chain reaction (PCR).

A major disadvantage of the culturing steps which are necessary before the bacteria can be identified is their time-consuming character. Some bacteria, such as *Mycobacterium tuberculosis*, require a few weeks' culture.

In principle, the PCR, involving an *in vitro* amplification, can be used to shorten the culturing steps or even render them superfluous. However, this requires that if species-specific primers are used, the bacterial species to be looked for in the sample be known beforehand because otherwise the species-specific primers will give a negative result.

By contrast, the use of universal primers, i.e. primers selected within evolutionarily conserved DNA sequences, gives a

positive result but no identification (see K. Chen, H. Neimark, P. Rumore, C.R. Steinman. 1989. Broad range DNA probes for detecting and amplifying eubacterial nucleic acids. FEMS Microbiology Letters 57:19-24).

Chen et al used DNA hybridization probes which are homologous to conserved parts of bacterial 16S ribosomal RNA (rRNA) sequences. The use of these 16S rRNA gene sequences has a number of advantages. The 16S rRNA gene is found in all bacteria. The gene consists of conserved parts, which are interrupted by species-specific sequences. Generally, several copies of the gene are present in the bacterial cell. As a result, a better/higher sensitivity can be achieved in proportion to non-ribosomal sequences.

Alternatively, the rRNA itself can be used as a basis for amplification. However, this requires that first a DNA copy of the rRNA be made. This can be done with the aid of a reverse transcriptase, such as avian myeloblastosis virus reverse transcriptase (AMV-RT), or a thermostable DNA polymerase which has this activity, for instance *Tth*-polymerase. This leads to a further improvement of the sensitivity, because up to 10,000 copies of the rRNA may be present in the bacterial cell.

The hybridization probes or primers used are directed against sequences which are not found in eukaryotes (hence not in humans either). Accordingly, these primers can be used for demonstrating the presence of unknown bacteria in a sample.

For a directed treatment of patients with e.g. antibiotics, however, it is necessary to know the identity of the bacteria. To identify the bacteria, either the nucleotide sequence will have to be determined or the amplified product of the PCR will have to be hybridized with a large number of species-specific DNA probes.

The first-mentioned method (determination of the nucleotide sequence) is time-consuming and costly.

The method utilizing specific probes which hybridize with the species-specific parts of the amplified rRNA sequence has been described for a (theoretical) PCR-based assay for the

determination of bacteremia by Roche Molecular Systems (D.U. Leong. 1992. Design of a PCR assay for the rapid detection of bacteremia. Infection in Medicine 7:43-48).

A disadvantage of this method is that one must have at one's disposal a large number of (tens of) species-specific probes to be able to identify the different species of bacteria. Accordingly, the system described by Leong has not yet been put into practice.

Rapid identification, for instance in the case of bacteremia or spotted fever, is often desirable and neither the known culturing method nor the current DNA technology provide any practical solution to this problem.

BRIEF SUMMARY OF THE INVENTION

The present invention provides a method for identifying a microorganism present in a sample, comprising

subjecting nucleic acid of the microorganism present in the sample to nucleic acid amplification utilizing one or more sets of universal primers which are based on a gene of the microorganism to be identified which comprises both conserved and variable regions, the primers having been selected in conserved regions which enclose a variable region;

bringing the product of the nucleic acid amplification into single-stranded form;

subjecting the amplification product brought into single-stranded form to an electrophoresis which is capable of separating single-stranded nucleic acids of equal length from each other on the basis of differences in nucleotide sequence;

detecting the electrophoresed nucleic acid; and

comparing the position of the electrophoresed nucleic acid with those of a set of reference nucleic acids of known microorganisms.

The microorganisms can be bacteria, viruses, fungi, actinomycetes or unicellular parasites but preferably consist of bacteria.

In the case of the identification of bacteria, the sets of universal primers are preferably based on the 16S rRNA gene or the 23S rRNA gene of bacteria. A highly suitable set of primers is based on the regions 1173-1192 and 1370-1389 of the 16S rRNA gene and then preferably consists of the primers ER1/ER2:

ER1: AGG CCC GGG AAC GTA TTC AC [SEQ ID NO:1], and

ER2: GAG GAA GGT GGG GAT GAC GT [SEQ ID NO:2].

Another highly suitable set of primers is based on the regions 103-119 and 341-357 of the 16S rRNA gene and then consists preferably of the primers ER3/ER4:

ER3: GGC GGA CGG GTG AGT AA [SEQ ID NO:3], and

ER4: ACT GCT GCC TCC CGT AG [SEQ ID NO:4].

Also useful are the following primer combinations ER5/ER6 and ER7/ER8:

ER5: GCA ACG CGA AGA ACC TTA CC [SEQ ID NO:5], and

ER6: AGC CAT GCA GCA CCT GTC TC [SEQ ID NO:6], based on the regions 966-985 and 1041-1060 of the 16S rRNA gene; as well as

ER7: GGA ACT GAA ACA TCT AAG TA [SEQ ID NO:7], and

ER8: CGG TAC TGG TTC ACT ATC GG [SEQ ID NO:8], based on the regions 188-207 and 444-463 of the 23S rRNA gene.

It is preferred, with a view to optimum specificity and differentiation between different species of bacteria, that at least two sets of primers be used in the nucleic acid amplification. This plurality of sets of primers can be used simultaneously in a multiplex amplification. A highly suitable multiplex PCR is obtained when the primer sets ER1/ER2 and ER3/ER4 are combined in one PCR incubation. Because the subsequent analysis takes place in one lane of the electrophoresis gel, a larger number of bands are obtained, the advantage being a better discrimination between the different bacterial species.

A plurality of sets of primers can also be used in a 'nested' nucleic acid amplification where a first amplification with a first set of primers is followed by a second amplification

with a second set of primers, the primers of the second set being based on regions located between those of the first set of primers. Such a 'nested' nucleic acid amplification leads to an improved detection limit (sensitivity).

The nucleic acid amplification preferably consists of a polymerase chain reaction (PCR) using a DNA polymerase, more particularly a thermostable DNA polymerase, such as *Taq*-polymerase, Vent-polymerase, *Tth*-polymerase, or Super*Taq*-polymerase (Super*Taq* is a tradename). Preferably, the DNA polymerase is subjected to a pretreatment by which contaminating DNA and/or RNA is removed.

The target nucleic acid in the PCR consists of DNA, viz. genomic DNA of the microorganism (such as the 16S rRNA gene) or cDNA, synthesized by reverse transcription of RNA of the microorganism (such as cDNA synthesized from the 16S rRNA itself).

However, the nucleic acid amplification can also be carried out according to a transcription-based amplification system (TAS), such as according to a self-sustained sequence replication (3SR) reaction, a nucleic acid system based amplification (NASBA) or a template mediated amplification (TMA). The target nucleic acid can in that case consist of RNA or DNA of the microorganism.

Methods according to the transcription-based amplification system (TAS) comprise a DNA synthesis step and an RNA transcription step. In the method an oligonucleotide primer containing a polymerase binding site (a promoter) is hybridized with a target RNA molecule or a denatured target DNA molecule. After the hybridization of the primer on the target molecule, a cDNA strand is synthesized by reverse transcription with the aid of reverse transcriptase. After denaturation (through heating) a second oligonucleotide is hybridized with the newly synthesized cDNA. By adding reverse transcriptase (or DNA polymerase) again, a double-stranded DNA molecule is synthesized. By adding an RNA polymerase, RNA copies are subsequently generated. Four cycles of

this process are sufficient to realize a million-fold amplification.

The self-sustained sequence replication (3SR) method is a modification of TAS. The most important difference is that the 3SR method is performed isothermally (37-42°C) and that the RNA target is broken down. RNase H is used to break down the RNA in the RNA-cDNA hybrid molecule (formed by the reverse transcriptase) and thereby to enable a conversion of the cDNA into a double-stranded DNA molecule. As a particular aspect, here a promoter sequence is also provided in the second primer, so that transcription can proceed from two terminal ends of the double-stranded DNA molecule. The RNA formed in this reaction is converted again into a RNA-cDNA hybrid molecule, so that the reaction sustains itself. In 15 minutes an approximately 100,000-fold RNA amplification is realized.

For the purpose of detecting the amplified and electrophoresed nucleic acid, labeled primers or labeled nucleotides can be utilized during the nucleic acid amplification. For instance, radioactively labeled primers or nucleotides can be utilized, or primers labeled with a fluorochrome, a chemiluminescent substance, biotin or digoxigenin. Highly suitable are primers labeled with fluorescein isothiocyanate (FITC) at the 5' end, in particular the primers ER1, ER2, ER3 and ER4.

The amplified and electrophoresed nucleic acid, if it is not labeled, can be detected by staining, for instance by silver staining, ethidium bromide staining or Stains-all staining.

For the electrophoresis of single-stranded nucleic acid, use is preferably made of a polyacrylamide gel electrophoresis under non-denaturing conditions.

If the product of the amplification consists of double-stranded DNA, it is brought into single-stranded form, for instance through heating, before it is subjected to the electrophoresis mentioned. If the product of the amplification

consists of single-stranded DNA or RNA, it can be subjected directly to the electrophoresis mentioned.

The nucleic acid of the microorganism present in the sample is preferably isolated from the sample before it is subjected to nucleic acid amplification. The sample can for instance consist of a clinical sample, such as urine, feces, sputum or blood, or a food.

The invention also provides an aid suitable for use in the new method according to the invention, comprising a set of electrophoresis patterns of nucleic acids of microorganisms serving as reference.

The invention further provides a set of aids suitable for use in the new method according to the invention, comprising

a nucleic acid amplification kit with one or more sets of universal primers which are based on a gene of the microorganism to be identified which comprises both conserved and variable regions, the primers being selected in conserved regions which enclose a variable region;

an electrophoresis kit for an electrophoresis which is capable of separating single-stranded nucleic acids of equal lengths from each other on the basis of differences in nucleotide sequence;

means for detecting labeled or unlabeled electrophoresed nucleic acid; and

a set of electrophoresis patterns of nucleic acids of microorganisms serving as reference.

DETAILED DESCRIPTION OF THE INVENTION

The present invention employs PCR (or a different nucleic acid amplification method) using suitably chosen universal primers, combined with an electrophoresis of the amplification product, in which sequence-dependent differences in mobility ("Sequence Dependent Differences in Mobility" [SDDM]) of single-stranded DNA (ssDNA) or RNA occur.

Incidentally, a similar method, the PCR-single-stranded conformation polymorphism method [PCR-SSCP] has recently been described as a possible method for demonstrating point-mutations (M. Orita, H. Iwahana, H. Kanazawa, K. Hayashi, T. Sekiya. 1989. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proceedings National Academy or Sciences USA 86:2766-2770).

PCR-SSCP has been designed for demonstrating a single point mutation. The PCR-SSCP looks at a small change in the mobility of the ssDNA with the point mutation with respect to 'wild-type' ssDNA. This technique is used for demonstrating a single point mutation in oncogenes and in hereditary diseases.

The DNA sequence which contains a possible point mutation is first amplified by means of the PCR. The amplified product is then denatured to form two single-stranded DNAs and analyzed by means of non-denaturing polyacrylamide gel electrophoresis.

Under non-denaturing conditions the DNA has a secondary structure which is the result of the nucleotide sequence and the composition of the solution in which the DNA is present. However, a change in the nucleotide composition need not always result in a change in the secondary structure. The mobility of the ssDNA during gel electrophoresis depends on the secondary structure formed. It is not to be predicted how the secondary structure influences the mobility. However, the expected change in mobility is slight because the length of the ssDNA fragment plays a dominant role in determining the mobility.

In a number of cases, therefore, it is impossible to find a difference in mobility despite the presence of a point mutation (K. Hayashi. 1992. PCR-SSCP: A method for detection of mutations. Genetic Analysis Techniques and Applications 9:73-79).

According to the invention, such an electrophoresis step is used for identifying (i.e. determining) bacterial species, not for demonstrating a single point mutation as happens in PCR-SSCP.

The principle of the present method (PCR-SDDM) for identifying bacteria is as follows. By means of universal primers

selected in conserved regions of the 16S rRNA gene, a DNA sequence is amplified which encloses a species-specific sequence. The amplified DNA is denatured (brought into single-stranded form) and subjected to non-denaturing gel electrophoresis.

It is supposed that the species-specific sequence gives rise to different secondary structures and mobilities in the gel. By determining the mobility of a particular ssSNA relative to one or more reference DNA molecules or markers and relating it to a data base with the mobility of ssDNAs coming from known bacterial species, the identity of the unknown bacterium can be determined.

In contrast with the (classic) PCR-SSCP, the present method does not look at the presence or absence of a single point mutation by determining a slight change in mobility of a ssDNA molecule as a result of this mutation, but at the mobility of an ssDNA molecule (in principle a random one) relative to a set of markers to subsequently determine the identity of a bacterium on the basis of this mobility.

Detection methods

For detecting the electrophoresed DNA or RNA, a staining technique can be used, for instance a silver staining or an ethidium bromide staining. In addition to these staining methods, a large number of other stainings for demonstrating DNA or RNA in gels have been described in the literature. A number of these methods are also suitable for demonstrating ssDNA in PCR-SDDM.

An alternative staining is with Stains-all [1-ethyl-2-(1-ethylnaphtho[1,2-d]thiazolin-2-ylidene)-2-methylpropenyl] naphtho[1,2-d]thiazolium bromide; Sigma, St. Louis, MO).

Direct labeling of the PCR product is possible through the use of radioactivity, fluorochromes, chemiluminescence labels, biotin and digoxigenin systems.

If the PCR reaction is performed in the presence of a suitable radioactive label such as ^{35}S, ^{32}P, ^{33}P deoxynucleotide triphosphates (dNTP) or ^{32}P, ^{33}P labelled primers, the ssDNA band pattern can be recorded with autoradiography or a β-scanner. The

autoradiogram can then be read into the computer with a scanner and be analyzed with suitable software. This enables direct assignment of a species name to an ssDNA band pattern. The data of a β-scanner can generally be inputted directly into the computer for analysis.

The use of chemiluminescent labels, for instance acridine esters, is possible only in combination with primers (so the primers are labeled, for the presence of the label has an influence on the mobility). The chemiluminescence is recorded either with a film or by means of a camera system. In both cases the data can be analyzed with computer equipment.

Labeling the primers with a fluorochrome, for instance FITC (the fluorochrome has an influence on the mobility of the ssDNA), has important advantages. Electrophoresis and analysis of the results can then be coupled by using an automatic DNA sequencer. The principle of an automatic DNA sequencer is that DNA molecules labeled with a suitable fluorochrome are electrophoresed through a polyacrylamide gel. At the lower end of the gel, the fluorochrome is irradiated with laser light of a suitable wavelength. The fluorescence intensity is determined and passed on to a computer. The time a DNA molecule needs to pass the laser is here a measure of the mobility.

The use of biotin and digoxigenin systems is possible but less practical because after the gel electrophoresis the DNA must be blotted to a nitrocellulose or nylon membrane. After blotting, the position of the DNA fragments is visualized by incubating the blot with a conjugate or an enzyme and either avidin or an anti-digoxigenin antibody, followed by the conversion of a substrate of the enzyme used in the conjugate.

The primers ER1, ER2, ER3 and ER4 also give excellent results when they are labeled with FITC at the 5'-end. Although the bands then display a different mobility, the resolution remains excellent or is even better. With the FITC-labeled primer combination ER1/ER2 it has been found possible to reproducibly distinguish *Enterobacter aerogenes* and *Enterobacter cloacae*,

whereas this sometimes gives rise to problems in the detection through silver staining. The same applies to *Listeria monocytogenes* and *Listeria ivanovii*.

The FITC-labeled primers are particularly suitable for the detection of the SDDM patterns with the aid of an automatic DNA sequencer.

Alternatives during and before the PCR

PCR amplification can be performed not only with *Taq*-polymerase but also with other thermostable DNA polymerases, for instance Vent-polymerase, *Tth*-polymerase, Super*Taq*-polymerase. By using other preparations with thermostable DNA polymerases it is often possible to prevent contamination of the polymerase preparation with DNA or RNA originating from the bacterial cell used for isolation of the polymerase. In such cases, if any, it will be necessary to treat the preparation with the thermostable DNA polymerase with DNase or RNase to remove contaminating DNA or RNA. DNase is then inactivated through a heat treatment. The RNase can be inactivated by the addition of an inhibitor such as RNasine.

The differentiation between different bacterial species (specificity) can be improved by using a second set of primers. In principle, it is possible to use both sets of primers simultaneously (multiplex PCR), so that two or more DNA fragments are amplified simultaneously. The primer sets should not be located unduly close to each other because that may give rise to amplification products resulting from the combination of primers from one set with primers of the other set. The amplification products should not be unduly short because otherwise double-stranded amplification product obtained with one primer set might, in the gel electrophoresis, overlap single-stranded product obtained with the second primer set. Nor should the amplification products be unduly long because they might then affect the desired secondary structures too much.

The detection limit of the system (sensitivity) can be improved by performing a so-called "nested PCR". In this set-up also two sets of primers are used. The second set of primers then lies between the positions of the primers in the first set. The first set is used during, for instance, 30 cycles, and a part of the PCR sample is then used for a second amplification of 30 or 35 cycles with the second set of primers.

Instead of DNA it is also possible to use RNA as target sequence. The advantage of using the rRNA is that up to 10,000 copies per cell can be present. With the aid of reverse transcriptase, the rRNA is then converted into a cDNA which subsequently serves as starting material for the above-described PCR methods.

An alternative method, however, is the 3SR method (T.R. Gingeras, K.M. Whitfield, D.Y. Kwoh. 1990. Unique features of the self-sustained sequence replication (3SR) reaction in the *in vitro* amplification of nucleic acids. Ann. Biol. Clin. 48: 498-501; J.C. Guatelli, K.M. Whitfield, D.Y. Kwoh, K.J. Barringer, D.D. Richman, T.R. Gingeras. 1990. Isothermal, *in vitro* amplification of nucleic acids by a multi-enzyme reaction modeled after retroviral replication. Proceedings National Academy of Sciences USA 87:1874-1878) and the "nucleic acid system based amplification" (NASBA) (T. Kievits, B. van Gemen, D. van Strijp, R. Schukking, M. Dircks, H. Adriaanse, L. Malek, R. Sooknanan, P. Lens. NASBA™ isothermal enzymatic in vitro nucleic acid amplification optimized for the diagnosis or HIV-1 infection. 1991. Journal or Virological Methods 35:273-286).

The principle of 3SR and that of the NASBA are comparable and both are derived from the transcription-based amplification system (TAS) (D.Y. Kwoh, G.R. Davis, K.M. Whitfield, H.L. Chapelle, L.J. Di Michele, T.R. Gingeras. 1989. Transcription-based amplification system and detection of amplified human immunodeficiency virus type 1 with a bead-based sandwich hybridization format. Proceedings National Academy of Sciences USA 86:1173-1177).

In summary, the principle of the NASBA is as follows. By means of a specific primer P1, which has a T7 promoter sequence at the 5'-end, a double-stranded DNA-RNA hybrid is made of the target RNA molecule using AMV-reverse transcriptase (AMV-RT). RNase H then breaks down the RNA strand of this hybrid. The second specific primer P2 can hybridize (anneal) with the ss-cDNA formed and the DNA-dependent DNA polymerase activity of AMV-RT makes the ss-cDNA double-stranded. The product is a double-stranded DNA molecule with a T7 promoter. The use of T7 RNA polymerase gives rise to a 100 to 1000-fold increase of the specific RNA. This RNA in turn can be used by AMV-RT for generating new cDNA molecules. This gives rise to a cyclic phase and an enormous increase in the amount of RNA. The entire process takes place at, for instance, 41°C. The RNA product is finally detected and analyzed. With a few small modifications, double-stranded DNA instead of RNA can serve as starting material.

A possible additional advantage of the NASBA system is that the RNA is used for SDDM identification. For SSCP it has been described that the use of RNA leads to greater differences in mobility (G. Sarkar, H.-S. Yoon, S.S. Somer. 1992. Screening for mutations by RNA single-strand conformation polymorphism (rSSCP): comparison with DNA-SSCP. Nucleic Acids research 20:871-878).

Detection of other microorganisms

Although the most important application of the invention will consist in identifying and discriminating between bacterial species, the invention is also useful for discriminating between genera or strains.

In addition to being suitable for the identification of bacteria, the method is in principle also suitable for the identification of fungi (actinomycetes) and unicellular parasites. The universal primers desribed herein, however, cannot be used because eukaryotes lack the species-specific part of the rRNA chosen for the bacteria. Fungi have no insertions in the rRNA sequence compared with human (mammalian) rRNA. On the other hand, species-specific sequences can be indicated which are flanked by conserved sequences, where universal eukaryotic primers can be selected. Contamination of the samples by human rRNA or rRNA DNA, however, leads to additional products in the PCR-SDDM, which may affect the interpretation of the data. For some parasites, such as *Plasmodium* (the causer of malaria), unique insertions in the rRNA have been described, but in such circumstances it is more obvious to look with *Plasmodium* specific primers. Here too, therefore, the above-mentioned universal eukaryotic primers will have to be used.

The use of the present method with viruses is rendered more difficult because of the strong heterogeneity of viruses. They generally do not have any common sequences. Exceptions are enteroviruses, for which universal primers have been described.

However, in the case of viruses the present method *can* be used for (epidemiologically) typefying isolates, for instance for discriminating between different serotypes of adenovirus, human papilloma virus (HPV) and human immunodeficiency virus types 1 and 2 (HIV). In such cases, species-specific primers will have to be used which enclose strain-specific sequences.

Quantitative PCR-SDDM

The PCR-SDDM can be adapted for quantitatively determining the amount of DNA or RNA in a sample and hence the number of microorganisms that are present. This may for instance be important in the quality assurance of water or for monitoring anti-microbial therapy. To this end, before the PCR step a known amount of DNA or RNA must be added. However, the PCR product should have a different mobility in the gel than the DNA to be quantified. By measuring the obtained signal (radioactivity: measuring α, β or γ-radiation or densitometry on the autoradiogram; fluorochrome: fluorescence intensity; silver staining and ethidium bromide: densitometry) and relating it to the signal obtained with known amounts of DNA or RNA which are used for the PCR, the amount of DNA or RNA and hence the number of microorganisms present in the sample can be determined.

Example 1

Sample preparation when using loose colonies or pure cultures

Gram-negative bacteria were cultured overnight on blood agar plates at 37°C, then scraped off the plates and lysed in deionized water with 5-10% chelex 100 (Biorad, Richmond, CA) by heating at 95°C for 5 minutes.

Gram-positive bacteria were lysed in 0.01% sodium dodecyl sulfate (SDS) and 5-10% chelex 100 by heating at 95°C for 5 minutes. After the lysis 0.5% Nonidet P-40 (Sigma, St. Louis, MO) was added to prevent inhibition of the *Taq*-polymerase by SDS.

Blood sample preparation for detecting bacteremia using the PCR

The sample preparation consisted of lysis of the blood cells followed by a filter concentration of any bacteria present. With the aid of this method (relatively) large volumes of blood could be lysed. At least 2 ml blood could be worked up by this method.

EDTA or citrate blood was diluted 1:1 with 1% Nonidet P40 (NP40; Sigma, St. Louis, MO) and frozen. After defrosting the

sample was centrifuged for 5 minutes at 4000 x g and a temperature of +4°C. The pellet was washed once with 0.5% NP40, and then centrifuged for 5 minutes at 4000 x g, followed by washing once with physiological salt and subsequent centrifugation at 4000 x g for 5 minutes. The pellet obtained was resuspended in 200 µl 1 x PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (weight/volume) gelatin) and incubated with 300 µg DNase I at 37°C for 15 minutes. Then the suspension was filtered through a 0.22 µm Durapore membrane filter (GVHP filter) (Millipore, Bedford, MA), and the filter was then washed twice with physiological salt. The filter was transferred to a 0.5 ml Eppendorf tube and any bacteria present were lysed in 50 µl 5%-10% chelex in deionized water by heating at 95°C for 10 minutes. After a centrifugation of 30 seconds at about 12,000 x g, 20 µl of the supernatant was used for PCR amplification.

PCR

The PCR was carried out for 35 cycles with primers directed against conserved 16S and 23S rRNA gene sequences (Chen et al. 1989, FEMS Microbiology Letters 57: 19-24, and G. van Camp, S. Chapelle and R. de Wachter. Amplification and sequencing of variable regions in bacterial 23S ribosomal RNA genes with conserved primer sequences. Current Microbiol. 1993, 27: 147-151).

Four primer sets were used. Primer sets ER1/ER2, ER3/ER4 and ER5/ER6 are directed against 16S rRNA gene sequences and primer set ER7/ER8 is directed against 23S rRNA gene sequences.
ER1: 5'-AGG CCC GGG AAC GTA TTC AC (nucleotide nos. 1173-1192) and
ER2: 5'-GAG GAA GGT GGG GAT GAC GT (complementary to nucleotide nos. 1370-1389);
ER3: 5'-GGC GGA CGG GTG AGT AA (nucleotide nos. 103-119) and
ER4: 5'-ACT GCT GCC TCC CGT AG (complementary to nucleotide nos. 341-357);

ER5: 5'-GCA ACG CGA AGA ACC TTA CC (nucleotide nos. 966-985) and ER6: 5'-AGC CAT GCA GCA CCT GTC TC (complementary to nucleotide nos. 1041-1060);

ER7: 5'-GGA ACT GAA ACA TCT AAG TA (nucleotide nos. 188-207) and ER8: 5'-CGG TAC TGG TTC ACT ATC GG (complementary to nucleotide nos. 444-463).

Each cycle consisted of 1 minute at 94°C, 1 minute at 55°C, 10 seconds at 72°C. The PCR reaction mixture (50 µl) consisted of:

either 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (weight/volume) gelatin, 100 µM of each primer, 1 U *Taq*-polymerase (Perkin-Elmer Cetus) and 100 µM of each dNTP (dATP, dCTP, dGTP, dTTP);

or 50 mM Tris-HCl (pH 9.0), 50 mM KCl, 7 mM $MgCl_2$, 2 mg/ml bovine serum albumin, 16 mM $(NH_4)_2SO_4$, 100 µM of each primer, 0.1 U Super-*Taq* polymerase (HT Biotechnology, Cambridge, UK) and 100 µM of each dNTP (dATP, dCTP, dGTP, dTTP).

Amplification of the target DNA resulted in a DNA fragment of about 218 bp for primer set ER1/ER2, 255 bp for primer set ER3/ER4, 95 bp for primer set E5/ER6, and 276 bp for primer set ER7/ER8 (small differences depending on bacterial species are possible).

Multiplex PCR

The PCR was carried out for 35 cycles with primers directed against conserved 16S rRNA gene sequences with primer sets ER1/ER2 and ER3/ER4.

Each cycle consisted of 1 minute at 94°C, 1 minute at 55°C, 10 seconds at 72°C. The PCR reaction mixture (50 µl) consisted of:

either 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (weight/volume) gelatin, 100 µM of each primer, 1 U *Taq*-polymerase (Perkin-Elmer Cetus) and 100 µM of each dNTP (dATP, dCTP, dGTP, dTTP);

or 50 mM Tris-HCl (pH 9.0), 50 mM KCl, 7 mM $MgCl_2$, 2 mg/ml bovine serum albumin, 16 mM $(NH_4)_2SO_4$, 100 µM of each primer, 0.1 U Super-*Taq* polymerase (HT Biotechnology, Cambridge, UK) and 100 µM of each dNTP (dATP, dCTP, dGTP, dTTP).

Amplification of target DNA resulted in a DNA fragment of about 218 bp for primer set ER1/ER2 and 255 bp for primer set ER3/ER4 (small differences depending on bacterial species are possible).

Analysis by means of gel electrophoresis

After amplification 5-10 µl of the PCR mixture was added to 5 µl sequencing sample buffer (95% formamide, 5 mM EDTA, 0.05% bromphenol blue and 0.05% xylene cyanol FF) and heated at 95°C for 5 minutes. Then the denatured DNA was directly placed on ice and applied to a gel for analysis after 10 minutes.

Analysis was performed with three different types of polyacrylamide gels. In all cases 10% glycerol was added to improve resolution. The gel types used are:

- 6% bisacrylamide/acrylamide (1:29), 0.05% ammonium persulfate and 0.005% TEMED in 54 mM Tris, 54 mM borate, 1.2 mM EDTA (0.6xTBE pH 8.3).
- 6% hydrolink longranger gel (JT Baker, Deventer, Netherlands), 0.05% ammonium persulfate and 0.005% TEMED in 54 mM Tris, 54 mM borate, 1.2 mM EDTA (0.6xTBE pH 8.3).
- 0,5 x MDE (Mutation Detection Enhancement) gel (JT Baker), 0.05% ammonium persulfate and 0.005% TEMED in 54 mM Tris, 54 mM borate, 1.2 mM EDTA (0.6xTBE pH 8.3).

The best results were obtained when using 0.5 x MDE gel, 0.6 x TBE and 10% glycerol.

When using polyacrylamide gels and longranger gels, a better resolution was obtained with spacers of 0.4 mm than with spacers of 0.75 mm.

Electrophoresis was performed at room temperature with 0.6 x TBE buffer at 1.5 W continuous output for 1.5-2 hours for a 6 x 8 cm gel and at 5 W continuous output for 3-4 hours for a 20

x 20 cm gel. For a 20 x 20 cm gel, also a period of 15 hours at 2W was used, so that the gel electrophoresis could take place overnight. The separation under these conditions was excellent.

After the electrophoresis the position (a measure for the mobility) of the ssDNA in the gel was determined with ethidium bromide staining or silver staining.

The ethidium bromide staining was carried out as follows. The gel was immersed for 5 minutes in a solution of 1 µg/ml ethidium bromide in 0.6 x TBE buffer followed by excess removal in 0.6 x TBE for 15 min. DNA bands were visualized with UV light.

In the silver staining the gels were first fixed in 50% methanol at 37°C for 30 minutes and then washed twice with deionized water. The gels were treated with 0.1% silver nitrate, 0.056% NaOH and 0.375% NH_4OH at 37°C for 15-30 minutes. Color development occurred through incubation with 0.005% citric acid and 0.019% formaldehyde. The gels were washed with deionized water and the color development was stopped with 50% methanol and 5% acetic acid.

According to an alternative silver staining method the gels were first fixed in 10% acetic acid for 20 minutes and then washed three times with deionized water for 2 minutes. The gels were treated with silver nitrate (1 g/l), 1.5 ml 37% formic acid per liter for 30 minutes. The gel was washed with deionized water for 20 seconds. Color development occurred through 2-5 minute incubation with 30 g/l sodium carbonate, 1.5 ml 37% formic acid per liter and 2 mg/l $Na_2S_2O_3.5H_2O$. The color development was stopped by treating the gels with 10% acetic acid for 5 min. All incubations were carried out at room temperature.

Example 2: use of fluorescein isothiocyanate labeled primers followed by analysis with an automatic DNA sequencer

PCR

The PCR was performed for 35 cycles with primers directed against conserved 16S rRNA gene sequences. For this purpose the

two primer sets ER1/ER2 and ER3/ER4 were used, the 5'-ends of the primers being labeled with fluorescein isothiocyanate (FITC).

Each cycle consisted of 1 minute at 94°C, 1 minute at 55°C, 10 seconds at 72°C. The PCR reaction mixture (50 µl) consisted of:

either 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (weight/volume) gelatin, 100 µM of each primer, 1 U *Taq*-polymerase (Perkin-Elmer Cetus) and 100 µM of each dNTP (dATP, dCTP, dGTP, dTTP);

or 50 mM Tris-HCl (pH 9.0), 50 mM KCl, 7 mM $MgCl_2$, 2 mg/ml bovine serum albumin, 16 mM $(NH_4)_2SO_4$, 100 µM of each primer, 0.1 U Super-*Taq* polymerase (HT Biotechnology, Cambridge, UK) and 100 µM of each dNTP (dATP, dCTP, dGTP, dTTP).

Amplification of target DNA resulted in a DNA fragment of about 218 bp for primer set ER1/ER2 and 255 bp for primer set ER3/ER4 (small differences depending on bacterial species are possible).

The primer combinations ER1/ER2 and ER3/ER4 can be combined in one PCR incubation (multiplex PCR). The analysis accordingly takes place in one lane of the polyacrylamide gel. This increases the number of bands and thereby improves the discrimination between the different species.

Analysis by means of gel electrophoresis

After amplification 0.1-1 µl of the PCR mixture was added to 5 µl 95% formamide, 5 mM EDTA and 0.05% dextran blue and heated at 95°C for 5 min. Then the denatured DNA was directly placed on ice and applied to a gel for analysis after 10 minutes.

Analysis was carried out with two types of polyacrylamide gels. In alle cases 10% glycerol was added to improve the resolution. The gel types used are:

-6% hydrolink longranger gel (JT Baker), 0.05% ammonium persulfate and 0.005% TEMED in 54 mM Tris, 54 mM borate, 1.2 mM EDTA (0.6xTBE (pH 8.3)).

-0.5 x MDE (Mutation Detection Enhancement) gel (JT Baker), 0.05% ammonium persulfate and 0.005% TEMED in 54 mM Tris, 54 mM borate, 1.2 mM EDTA (0.6xTBE (pH 8.3)).
The use of 0.5xMDE gel gave a better resolution.

The gel thickness used was 0.5 mm.

Electrophoresis was carried out at 30°C with 0.6xTBE buffer at 30 W continuous output for 300 minutes for a 20 x 34 cm (l x b) gel and for 800 minutes for a 30 x 34 cm (l x b) gel. The electrophoresis was carried out on an ALF DNA sequencer (Pharmacia, Sweden). Instead of an ALF DNA sequencer, other automatic DNA sequencers can be used as well, such as automatic DNA sequencers of Applied Biosystems Inc.(CA, USA).

To enable a good analysis, two internal markers were added to each sample. These markers were used to correct the small local differences in electrophoresis conditions which are present in the gel. The markers consisted of a sequence coming from the gene for toxin B of *Clostridium difficile*. The size of the markers was chosen such that one marker has a mobility that is greater than that of the products obtained with PCR-SDDM and one marker has a mobility smaller than the PCR-SDDM products. (Note: of course, other DNA sequences meeting the above-mentioned conditions can also be used as markers).

In total, three markers were used: 150 nucleotides, 203 nucleotides and 257 nucleotides. The markers of 150 and 257 nucleotides were used in multiplex PCR-SDDM with primers ER1/ER2 and ER3/ER4. The markers of 150 and 203 nucleotides were used for PCR-SDDM with primer combination ER1/ER2 alone.

The markers are obtained with the aid of PCR. For that purpose the following primers were used (nucleotide numbers are derived from the toxin B gene sequence published by L.A. Barroso, S.Z. Wang, C.J. Phelps, J.L. Johnson, and T.D. Wilkins. Nucleic Acids Res. 1990, 18: 4004):

91.50 FITC-5'-GTC AGA GAA TAC TGT AGT CG (nucleotide nos. 508-527);

90.11 5'-TCC AAT CCA AAC AAA ATG TA (complementary to nucleotide nos. 691-710);
TOXB321r 5'-CTT TAG CTC TAA TAC TTC TG (complementary to nucleotide nos. 638-657);
TOXB428r 5'-CTA TTT ACA TCT TTC CAT TG (complementary to nucleotide nos. 755-764).
Here 91.50 with 90.11 gives the product of 203 nucleotides, 91.50 with TOXB321r the product of 150 nucleotides and 91.50 with TOXB428r the product of 257 nucleotides.

The following PCR conditions were used. A suspension of toxigenic *Clostridium diffcile* in water was heated at 95°C for 5 min. Ten µl of heated suspension was added to the PCR incubation mixture consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 100 µM of each of the four dNTPs, 50 pmol of each primer (in each case, only two primers were used, one of which was 91.50) and 1.0U *Taq*-polymerase. The PCR was carried out for 35 cycles. Each cycle consisted of 1 min 94°C, 1 min 55°C and 10 sec 72°C.

Used as marker was 0.2 µl of the incubation mixture in 5 µl 95% formamide, 5 mM EDTA and 0.05% dextran blue.

Results Example 1

The method utilizing primer set ER1/ER2 was applied to 114 bacterial strains selected from 40 species and 15 genera. This resulted in 35 different ssDNA band patterns, as summarized in Table 1.

Table 1: PCR-SDDM patterns with primers ER1-ER2 and silver staining

Pattern no.	Species (gram-positive)	tested number strains
1	*Clostridium difficile*	11
2	*Clostridium bifermentans*	1
3	*Clostridium butyricum*	1

	4	*Clostridium cadaveris*	1
	5	*Clostridium clostridiiforme*	1
	6	*Clostridium histolyticum*	1
	7	*Clostridium innocuum*	1
5	8	*Clostridium paraputrificum*	1
	9	*Clostridium perfringens*	1
	10	*Clostridium putrificum*	1
	11	*Clostridium sporogenes*	1
	12	*Listeria monocytogenes*	7
10	13	*Listeria innocua*	4
	14	*Listeria ivanovii*	3
	15	*Listeria grayi*	2
	16	*Staphylococcus aureus*	12
	17	*Staphylococcus epidermidis*	4
15	18	Group A hemolytic *Streptococcus*	2
	19	*Acinetobacter calcoaceticus*	2
	20	*Bacteroides fragilis*	2
	21	*Citrobacter freundii*	3
		Citrobacter amalonaticus	1
20	22	*Escherichia coli*	8
		Shigella boydii	1
		Shigella dysenteriae	1
		Shigella flexneri	1
		Salmonella enterica subsp. *enterica*	4
25	23	*Enterbacter aerogenes*	3
	24	*Enterobacter agglomerans*	4
	25	*Enterobacter cloacae*	5
	26	*Enterobacter gergoviae*	2
	27	*Enterobacter sakazakii*	1
30	28	*Klebsiella oxytoca*	2
	29	*Klebsiella pneumoniae*	3
	30	*Morganella morganii*	2
	31	*Proteus mirabilis*	3
		Proteus vulgaris	2
35	32	*Pseudomonas aeruginosa*	3

33	*Pseudomonas fluorescens*	1
34	*Pseudomonas malthophilia*	3
35	*Pseudomonas putida*	2

The ssDNA patterns were species-specific, except that the tested *Proteus* spp. gave the same pattern. The same result was also obtained with *Citrobacter freundii* and *Citrobacter amalonaticus*. This was also the case for *Escherichia coli / Salmonella enterica / Shigella* spp. group. The identical patterns between *Escherichia coli* and the *Shigella* spp. were not entirely unexpected because both species are about 99% identical at a genetic level and are considered to be of the same species by some (D.J. Brenner. Introduction to the family Enterobacteriaceae. In: A. Balows, H.G. Truper, M. Dworkin, W. Harder, K.H. Schleifer (eds.) The prokaryotes, 2nd ed. vol. III p.2683. Springer-Verlag, N.Y.). The similarity between *Escherichia coli* and *Salmonella enterica* was less expected, although the two species are very closely related.

The use of a second primer set (ER3/ER4) largely solved these problems (see Table 2).

Table 2: PCR-SDDM patterns with primers ER3-ER4 and silver staining

Pattern no.	Species	tested number of strains
1	*Citrobacter amalonaticus*	1
2	*Citrobacter freundii*	2
3	*Escherichia coli*	5
4	*Salmonella enterica* susp. *enterica*	4
5	*Shigella flexneri*	2
	Shigella sonnei	1
	Shigella dysenteriae	2
6	*Proteus mirabilis*	4
7	*Proteus vulgaris*	2

By using primer set ER3/ER4 it is possible to discriminate *Proteus mirabilis* from *Proteus vulgaris* and *Citrobacter freundii* from *Citrobacter amalonaticus*. Within the *Escherichia coli / Salmonella enterica / Shigella* spp. group, a distinction could be made between *Escherichia coli*, *Salmonella enterica* and the *Shigella*. Within the *Shigella* spp. no distinction could be made.

The primer sets ER1/ER2 and ER3/ER4 can be combined in a multiplex PCR, so that with a single PCR-SDDM analysis the results of the two primer sets can be combined. This does not lead to any loss of discriminative ability.

Although the primer combinations ER5/ER6 and ER7/ER8 give useful results, the best results were obtained with ER1/ER2 and/or ER3/ER4.

Results Example 2

SSDM-PCR with FITC-labeled ER1/ER2 primers followed by analysis on an automatic DNA sequencer was applied to 57 bacterial strains selected from 14 species and 7 genera. This resulted in 12 different ssDNA band patterns as summarized in Table 3.

Table 3: PCR-SDDM patterns with FITC labeled primers ER1-ER2 and analysis on an automatic sequencer

Pattern no.	Species	tested number of strains
1	*Enterbacter aerogenes*	1
2	*Enterobacter cloacae*	2
3	*Escherichia coli*	29
	Salmonella enterica susp. *enterica*	2
4	*Listeria monocytogenes*	2
5	*Listeria innocua*	2
6	*Listeria ivanovii*	2
7	*Listeria grayi*	2
8	*Klebsiella oxytoca*	2
9	*Klebsiella pneumoniae*	2
10	*Proteus mirabilis*	2
	Proteus vulgaris	1
11	*Staphylococcus aureus*	6
12	*Staphylococcus epidermidis*	2

The ssDNA band patterns were species-specific, except that the tested *Proteus* spp. gave the same pattern. This was also the case for *Escherichia coli / Salmonella enterica*. (These results correspond with the results obtained with the method described in Example 1.)

SSDM-PCR with the FITC-labeled ER1/ER2 and ER3/ER4 primer sets followed by analysis on an automatic DNA sequencer was applied to 41 bacterial strains selected from 10 species and 3 genera. This resulted in 10 different ssDNA patterns as summarized in Table 4.

Table 4: Multiplex PCR-SDDM patterns with FITC labeled primers ER1-ER2, ER3-ER4 and analysis on an automatic sequencer.

Pattern no.	Species	tested number of strains
1	*Escherichia coli*	2
2	*Listeria innocua*	3
3	*Listeria ivanovii*	3
4	*Listeria monocytogenes*	3
5	*Listeria grayi*	5
6	*Listeria seeligeri*	5
7	*Listeria welshimiri*	2
8	*Staphylococcus aureus*	9
9	*Staphylococcus epidermidis*	7
10	*Staphylococcus saprophyticus*	2

The ssDNA band patterns with the FITC-labeled primer sets ER1/ER2 and ER3/ER4 are found to be species-specific for the tested bacterial species.

From these results we conclude that it is possible to use the PCR-SDDM method described here for the identification of bacteria. For this, a limited number of primer sets are necessary which result in one or a few ssDNA band patterns for each species. However, the presence of large numbers of bacteria which do not have to be identified may interfere.

Note: At present there is an ongoing discussion about the nomenclature of the *Salmonella* spp. The WHO (World Health Organisation) adheres to *Salmonella enterica* while the American Society for Microbiology adheres to *almonella cholerasuis*.

SEQUENCE LIST

SEQ ID NO:1
LENGTH: 20 nucleotides
TYPE: nucleotides
STRANDEDNESS: single
AGGCCCGGGA ACGTATTCAC 20

SEQ ID NO:2
LENGTH: 20 nucleotides
TYPE: nucleotides
STRANDEDNESS: single
GAGGAAGGTG GGGATGACGT 20

SEQ ID NO:3
LENGTH: 17 nucleotides
TYPE: nucleotides
STRANDEDNESS: single
GGCGGACGGG TGAGTAA 17

SEQ ID NO:4
LENGTH: 17 nucleotides
TYPE: nucleotides
STRANDEDNESS: single
ACTGCTGCCT CCCGTAG 17

SEQ ID NO:5
LENGTH: 20 nucleotides
TYPE: nucleotides
STRANDEDNESS: single
GCAACGCGAA GAACCTTACC 20

SEQ ID NO:6
LENGTH: 20 nucleotides
TYPE: nucleotides
STRANDEDNESS: single
AGCCATGCAG CACCTGTCTC 20

SEQ ID NO:7
LENGTH: 20 nucleotides
TYPE: nucleotides
STRANDEDNESS: single
GGAACTGAAA CATCTAAGTA 20

SEQ ID NO:8
LEN : 20 nucleotides
TY nucleotides
ST DNESS: single
CG TGGT TCACT CGG 0

CLAIMS

1. A method for identifying a microorganism present in a sample, comprising:

subjecting nucleic acid of the microorganism present in the sample to nucleic acid amplification utilizing one or more sets of universal primers which are based on a gene of the microorganism to be identified which contains both conserved and variable regions, the primers being selected in conserved regions which enclose a variable region;

bringing the product of the nucleic acid amplification into single-stranded form;

subjecting the amplification product brought into single-stranded form to an electrophoresis which is able to separate single-stranded nucleic acids of equal lengths from each other on the basis of differences in nucleotide sequence;

detecting the electrophoresed nucleic acid; and

comparing the position of the electrophoresed nucleic acid with those of a set of reference nucleic acids of known microorganisms.

2. A method according to claim 1, wherein said microorganisms consist of bacteria, viruses, fungi, actinomycetes or unicellular parasites.

3. A method according to claim 1, wherein said microorganisms consist of bacteria.

4. A method according to claim 3, wherein said sets of universal primers are based on the 16S rRNA gene or the 23S rRNA gene of bacteria.

5. A method according to claim 4, wherein a set of primers is used based on the regions 1173-1192 and 1370-1389 of the 16S rRNA gene, such as the primer set

ER1: AGG CCC GGG AAC GTA TTC AC

ER2: GAG GAA GGT GGG GAT GAC GT

and/or a set of primers is used based on the regions 103-119 and 341-357 of the 16S rRNA gene, such as the primer set

ER3: GGC GGA CGG GTG AGT AA

ER4: ACT GCT GCC TCC CGT AG

and/or a set of primers is used based on the regions 966-985 and 1041-1060 of the 16S rRNA gene, such as the primer set

ER5: GCA ACG CGA AGA ACC TTA CC

ER6: AGC CAT GCA GCA CCT GTC TC

and/or a set of primers is used based on the regions 188-207 and 444-463 of the 23S rRNA gene, such as the primer set

ER7: GGA ACT GAA ACA TCT AAG TA

ER8: CGG TAC TGG TTC ACT ATC GG.

6. A method according to claim 1, wherein at least two sets of primers are used in the nucleic acid amplification.

7. A method according to claim 6, wherein said at least two sets of primers are used simultaneously in a multiplex amplification.

8. A method according to claim 6 or 7, wherein the primer sets ER1/ER2 and ER3/ER4 are used.

9. A method according to claim 6, wherein a 'nested' nucleic acid amplification is carried out whereby a first amplification with a first set of primers is followed by a second amplification with a second set of primers, the primers of the second set being based on regions located between those of the first set of primers.

10. A method according to claim 1, wherein the nucleic acid amplification consists of a polymerase chain reaction (PCR) utilizing a DNA polymerase.

11. A method according to claim 10, wherein use is made of a thermostable DNA polymerase.

12. A method according to claim 11, wherein *Taq*-polymerase, Vent-polymerase, *Tth*-polymerase, or Super*Taq*-polymerase is used.

13, A method according to claim 10, wherein said DNA polymerase is subjected to a pretreatment whereby contaminating DNA and/or RNA is removed.

14. A method according to claim 10, wherein the target nucleic acid consists of DNA selected from genomic DNA of the microorganism and cDNA synthesized by reverse transcription of RNA of the microorganism.

15. A method according to claim 1, wherein the nucleic acid amplification is carried out according to a transcription-based amplification system (TAS).

16. A method according to claim 15, wherein the nucleic acid amplification is carried out according to a self-sustained sequence replication (3SR) reaction, a nucleic acid system based amplification (NASBA) or a template mediated amplification (TMA).

17. A method according to claim 15, wherein the target nucleic acid consists of RNA or DNA of the microorganism.

18. A method according to claim 1, wherein, for the purpose of detecting the amplified and electrophoresed nucleic acid, labeled primers or labeled nucleotides are used during the nucleic acid amplification.

19. A method according to claim 18, wherein radioactively labeled primers or nucleotides are used during the nucleic acid amplification.

20. A method according to claim 18, wherein during the nucleic acid amplification, primers are used which are labeled with a fluorochrome, a chemiluminescent substance, biotin or digoxigenin.

21. A method according to claim 1, wherein the amplified and electrophoresed nucleic acid is detected by staining.

22. A method according to claim 21, wherein the detection of the amplified and electrophoresed nucleic acid is carried out by silver staining, ethidium bromide staining or Stains-all staining.

23. A method according to claim 1, wherein for said electrophoresis of single-stranded nucleic acid, use is made of a polyacrylamide gel electrophoresis under non-denaturing conditions.

24. A method according to claim 1, wherein the product of the amplification consists of double-stranded DNA, which is brought into single-stranded form by heating before it is subjected to said electrophoresis.

25. A method according to claim 1, wherein the product of the amplification consists of single-stranded RNA, which is subsequently subjected to said electrophoresis.

26. A method according to claim 1, wherein the nucleic acid of the microorganism present in the sample is isolated from the sample before it is subjected to nucleic acid amplification.

27. A method according to claim 1, wherein the sample consists of a clinical sample, such as urine, feces, sputum or blood, or a food.

28. An aid suitable for use in the method according to claim 1, comprising a set of electrophoresis patterns of nucleic acids of microorganisms serving as reference.

29. A set of aids suitable for use in the method according to claim 1, comprising:

a nucleic acid amplification kit with one or more sets of universal primers based on a gene of the microorganism to be identified which comprises both conserved and variable regions, the primers being selected in conserved regions which enclose a variable region;

an electrophoresis kit for an electrophoresis which is able to separate single-stranded nucleic acids of equal lengths from each other on the basis of differences in nucleotide sequence;

means for detecting labeled or unlabeled electrophoresed nucleic acid; and

a set of electrophoresis patterns of nucleic acids of microorganisms serving as reference.